

Basal cell subpopulation as putative human prostate carcinoma stem cells

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Abstract: The present study examines the expression of p63, glutathione S-transferase- π (GSTP1) and α -methylacyl-CoA-racemase (AMACR) in serial slices in proliferative inflammatory atrophy (PIA) in order to implicate that some of the basal cells are probably the putative human prostate carcinoma stem cells (PHPCSC). Archived tissue sections obtained after radical prostatectomy from cases (n=30) comprising of PIA were tested using immunohistochemistry with antibodies against AMACR (Dako), p63 and GSTP1 (Labvision) and visualized by biotin-streptavidin-peroxidase kit (DAKO LSAB 2 kit). Quantitative immunohistochemistry analysis (QIHC) of the studied antigen expression levels revealed that there are two populations of p63 basal cells. Type I basal cells had high AMACR, low GSTP1 and p63 expression. Type II basal cells had low AMACR, high GSTP1 and p63 expression. Therefore, we propose that the putative human prostate carcinoma stem cells probably reside within the population of type I basal cells.

Key words: p63 - GSTP1 - AMACR - PHPCSC - Quantitative immunohistochemistry

Introduction

Prostate cancer (PCa) is initially regarded as an androgen-dependent disease, as therapeutic androgen ablation induces massive apoptosis of malignant cells and clinically leads to tumor regression. However, after a remission period of up to several years, mutations occur and tumors usually progress despite low circulating androgen levels. Currently, no therapeutic options are available for the treatment of androgen-independent PCa [24,25]. If the prostate carcinoma stem cell have been targeted before these mutation occur, the progression to androgen independence might be prevented [18].

Several theories about the origin of putative human prostate carcinoma stem cells (PHPCSC) have been proposed and postulated suggesting that they probably arise from normal stem cells, transit-amplifying cells [3,26] or de-differentiated luminal cells [9,14].

New antigens as candidates for identifying these putative carcinoma stem cells are p63, GSTP1 and AMACR.

The nuclear protein p63, a homologue of the p53 tumor suppressor gene, is expressed in the basal cell compartment of the prostate gland tissue. p63-deficient mice showed agenesis of the prostate gland and severe defects in the development of other organs, suggesting that p63 is necessary for stem-cell maintenance [25].

GSTP1, encoding glutathione S-transferase- π , has been proposed to be a caretaker gene, protecting cells against genome damage mediated by oxidants and electrophiles from inflammation or dietary exposures [15].

It has been reported that the enzyme marker α -methylacyl-CoA-racemase (AMACR) has high specificity of expression in PCa (compared to benign processes in the prostate). The AMACR gene, encodes the enzyme α -methylacyl-CoA-racemase, which is localized in the cell mitochondria and peroxysoma, and plays a role in the fatty acid metabolism [10].

The present study examines the expression of p63, AMACR and GSTP1 in proliferative inflammatory atrophy (PIA), which is regarded as a precancerous state in order to explore the basal cell population for putative prostate carcinoma stem cells.

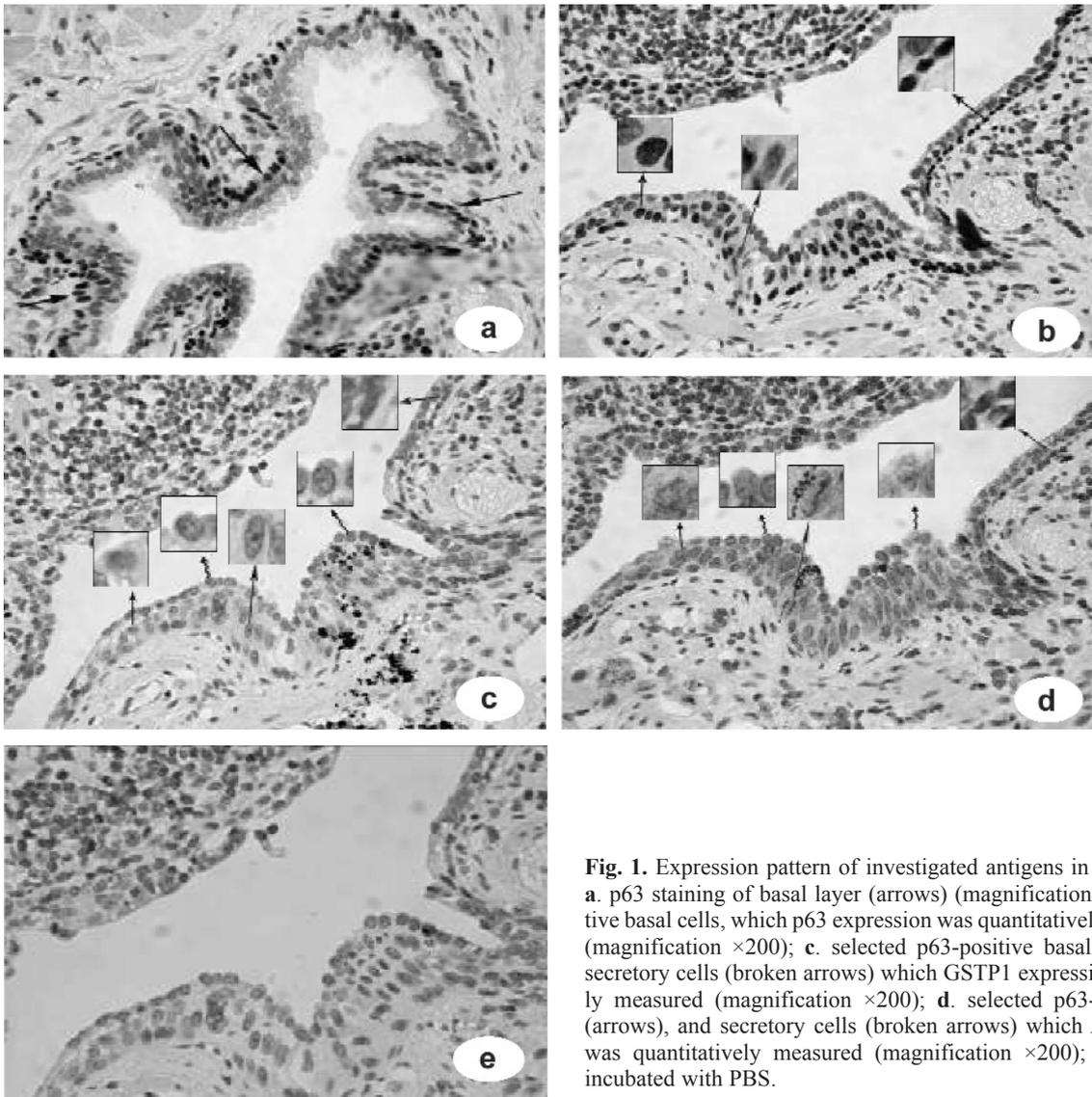


Fig. 1. Expression pattern of investigated antigens in serial slices in PIA; **a.** p63 staining of basal layer (arrows) (magnification $\times 200$); **b.** p63 positive basal cells, which p63 expression was quantitatively measured (arrows) (magnification $\times 200$); **c.** selected p63-positive basal cells (arrows), and secretory cells (broken arrows) which GSTP1 expression was quantitatively measured (magnification $\times 200$); **d.** selected p63-positive basal cells (arrows), and secretory cells (broken arrows) which AMACR expression was quantitatively measured (magnification $\times 200$); **e.** negative control incubated with PBS.

Materials and methods

Tissue specimens. Tissue samples were acquired after radical prostatectomy ($n=30$) from the periphery zone of the prostate of cases including all: PIA, prostate intraepithelial neoplasia (PIN) and PCa. The average age of the patients was 65 years (from 49 to 80 years old). The paraffin embedded tissue sections were processed and provided by the Pathology Department of the University Hospital, Pleven, Bulgaria and evaluated using conventional immunohistochemistry. Routine microscopy with hematoxylin-eosin staining was performed prior to the immunohistochemical investigation. The study was approved by the Ethics Committee of the hospital. Informed consent was asked and achieved from the patients in accordance with the Helsinki Declaration.

Antibodies. Polyclonal rabbit antibody against GSTP1, and mouse monoclonal antibody against p63 (clone 4A4) were obtained from LAB VISION (USA). Monoclonal rabbit anti-AMACR antibody, clone 13H4 was produced by DAKO (Dakocytomation, USA).

Immunohistochemistry. The tissue sections were tested using immunohistochemistry with antibodies against AMACR, p63,

GSTP1 on serial tissue section and visualized by peroxidase-based DAKO LSAB 2 kit (Dako, CA, USA).

Briefly, the paraffin sections were heated up to 57°C in thermostat and immersed in xylene three times for 10 min each and then hydrated through series of graded alcohols and then in PBS, $\text{pH}=7.2$. The sections treated that way were incubated with the corresponding antibodies for 2 hours at 30°C . After washing with PBS, the specific binding was detected by DAKO LSAB-2 SYSTEM, based on biotin-streptavidin-peroxidase, and visualized in pink color, using DAKO AEC substrate chromogen system (DAKO, CA, USA). Then, the sections were counterstained with hematoxylin (Sigma, USA) and all were embedded in aqueous mounting media (70% glycerol aqueous solution). Serial sections from the same samples but with no first antibody were used as negative controls and were incubated only with PBS and the DAKO LSAB-2 SYSTEM.

Quantitative immunohistochemistry (QIHC). Only parts incorporating PIA were analyzed for the expression of the investigated antigens by the basal cell layer cells as well as by the secretory ones above them. For each case up to 10 PIA lesions were analyzed with 5 distinct microscopic fields per each. Microphotographs were acquired at $\times 100$ and $\times 200$ as TIFF image files, using an

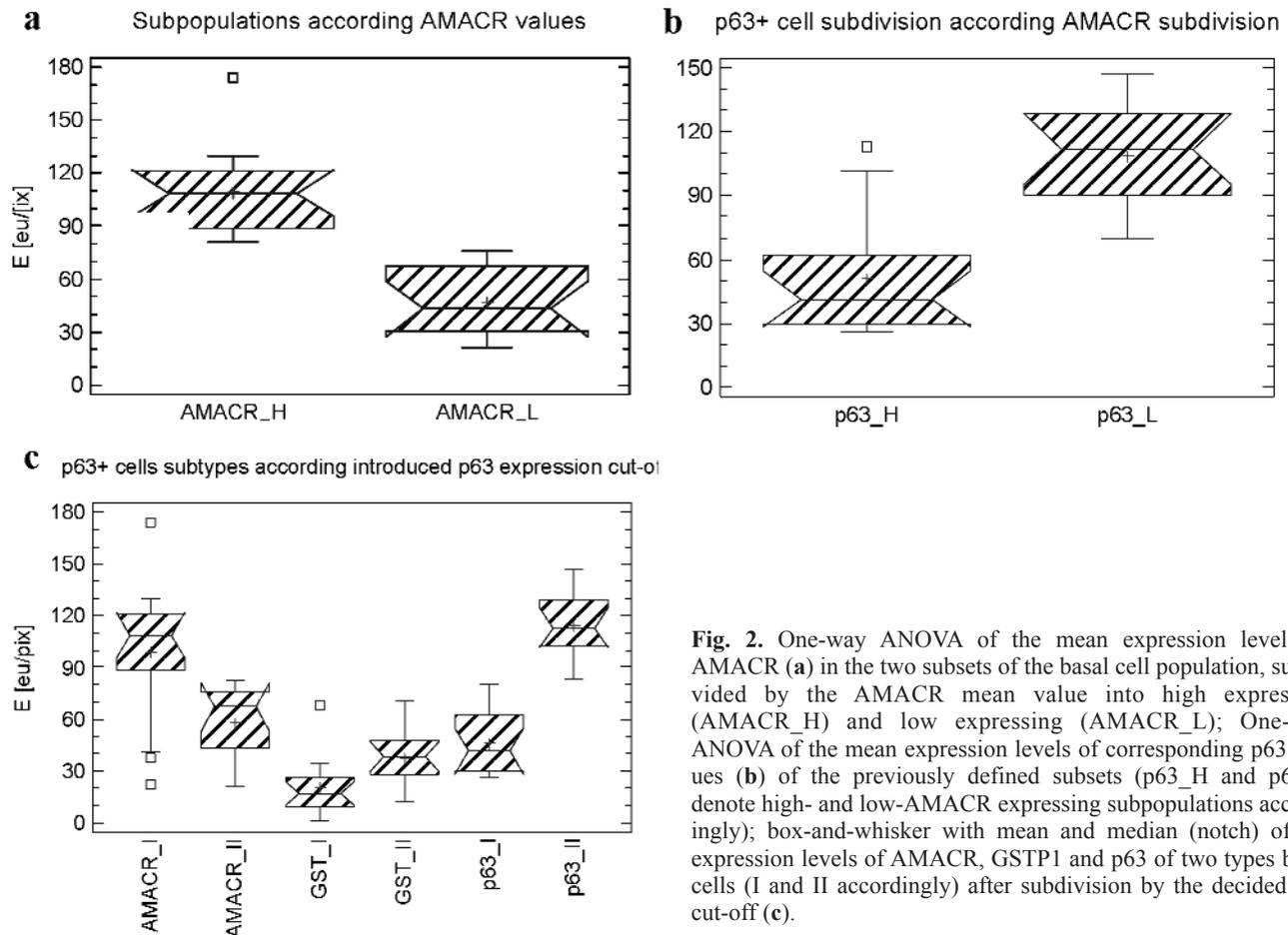


Fig. 2. One-way ANOVA of the mean expression levels of AMACR (a) in the two subsets of the basal cell population, subdivided by the AMACR mean value into high expressing (AMACR_H) and low expressing (AMACR_L); One-way ANOVA of the mean expression levels of corresponding p63 values (b) of the previously defined subsets (p63_H and p63_L denote high- and low-AMACR expressing subpopulations accordingly); box-and-whisker with mean and median (notch) of the expression levels of AMACR, GSTP1 and p63 of two types basal cells (I and II accordingly) after subdivision by the decided p63 cut-off (c).

Olympus CAMEDIA C-5050 (Olympus Corp., Japan) attached to an "Olympus BX-40" (Olympus Corp., Japan) microscope system. "GIMP 2" (The GNU image manipulation program) was used as image-processing software. The sub-images from serial tissue sections resembling identical cells were further extracted and the expression levels of the antigens were quantitatively determined.

Chromogen abundance was quantified by determining the cumulative signal strength of the digital image file of a histological relevant region of interest [11,12]. We applied our own modification [4] concerning the pixel selection algorithms as well as our in-house software for the image quantification. The amount of chromogen per pixel was determined by subtracting the mathematical energy (EM) of the control slide (i.e., not exposed to primary antibody) from that in the homologous region of the experimental slide (i.e., exposed to primary antibody). Chromogen quantity (EM) is expressed as energy units per pixel (eu/pix).

Statistical analysis. All data reported herein are valueless and are reported as energy units per pixel (eu/pix). Statistical evaluations were performed using "StatGraphics for Windows v.5" (Manugistics, USA). In all instances, data are expressed as the mean \pm SEM (standard error of mean).

Results

The localization of the basal layer in the examined PIA lesions was immunohistochemically determined using p63 basal nuclei staining (Fig. 1a). Then on sub-

images of the serial slices, the p63+ basal cells were selected and their corresponding p63 (Fig. 1b), GSTP1 (Fig. 1c) and AMACR (Fig. 1d) expressions were quantitatively assessed (QIHC). The GSTP1 and AMACR expressions for most of the overlaying secretory cells were evaluated as well (Fig. 1c and 1d).

Since AMACR expression is believed to strongly correlate with PCa malignancy [5], the level of tumor differentiation [7], and cancer-related death [19], we first evaluated its expression levels in the basal cells. Since there was statistically significant probability these data to come from normal distribution (*Chi-Square goodness-of-fit* = 6.70, $p=0.82$; *Shapiro-Wilks* $W=0.96$, $p=0.45$; both $p>0.10$) the cell population was subdivided according AMACR mean value into two categories: high- and low-expressing AMACR cells (Fig. 2a). When the p63 expression of the respective cells belonging to these two groups was compared using single factor ANOVA, there were statistically significant difference between their mean values (*F-ratio*=31.31, $p<0.00001$; additionally - *Kruskall-Wallis Test*: $p=0.0002$, Fig. 2b). Then, based on the span (mean and standard deviation) of these two sub-groups (high- and low-expressing AMACR), we determined a

Table 1. QIHC evaluation of the expression of p63, GSTP1 and AMACR in [eu/pix]. Two types of basal cells and one type secretory cells were observed. The data represents the mean values of the investigated groups \pm SEM (standard error of mean).

| Antigen expression [eu/pix] | | | | | | | |
|-----------------------------|------------------------|-----------------------|------------------------|--------------------|-----------------------|------------------------|--------------------|
| p63 | | GSTP1 | | | AMACR | | |
| Type I Basal cells | Type II Basal cells | Type I Basal cells | Type II Basal cells | Secretory cells | Type I Basal cells | Type II Basal cells | Secretory cells |
| 45.75+4.38 | 116+4.63 | 16.07+2.08 | 46.92+3.64 | 44.43+3.46 | 116+8.658 | 49.07+5.75 | 40+7.47 |

value of $\sim 82,0589$ eu/pix expression of p63 to be our cut-off value for subdividing further the basal cells into subtypes (Fig. 2c).

According to the antigen expression profiles based on all investigated antigens two types of basal cells, and one type secretory cells were observed (Table 1). Type I basal cells had low p63, GSTP1 and high AMACR expression. Type II basal cells had the same type of expression as secretory cells. Their p63 and GSTP1 expression was higher, and the AMACR expression was lower than type I basal cells. There was statistically significant difference in the staining intensities of p63, GSTP1 and AMACR among type I and type II basal cells ($p < 0.05$).

We further performed correlation analysis on the data for p63 basal cell subtype I and II with their respective AMACR and GSTP1 values and observed statistically significant moderately strong correlation {with $R^2=47.86\%$, $p < 0.01$ ($p=0.0061$) and $R^2=30.56\%$, $p < 0.01$ ($p=0.0403$) accordingly} between them only in the case of type I basal cells.

Discussion

The involvement of the different prostate cell types in prostate carcinogenesis remains unclear. Although prostate cancers are phenotypically and behaviorally similar in many respects to secretory cells (i.e., they express PSA, AR and are androgen dependent), recent studies suggest that PCa may arise from a more immature (intermediate) cell type located within the basal or luminal cell layer [2,3]. Evidence suggestive of this hypothesis comes from the identification of basal cell associated cytokeratins and genes in prostate cancer, particularly androgen-independent tumors [2]. In addition, it is hypothesized that prostate cancers, like other epithelial and non epithelial cancers, must arise from stem or progenitor cells, rather than from a terminally differentiated cell type [3]. However, the identity and localization of such cell types within the prostate is not known. Based on many lines of circumstantial evidence the consensus is that prostatic epithelial stem cells reside in the basal compartment [20,24].

Signoretti *et al.* [21] and Parsons *et al.* [17] have recently reported that p63 is a basal cell marker expressed by a majority of prostate epithelial cells in tissue culture. Although p63 expression is not restricted to stem cells, the highest expression of p63 occurs in stem cells and p63 has been suggested to be a marker of epithelial stem/progenitor cells [13,28].

In vitro studies have shown that the normal prostate stem cells lineages possess self-renewal ability and generate two distinct cell lineages - quiescent neuroendocrine cells and actively proliferating p63⁺/PSCA⁻/AR⁻ transit-amplifying cells [8]. The p63⁺ transit-amplifying cells lose their p63 expression and shift to express PSCA, becoming proliferatively quiescent intermediate cells [8,23]. These ones probably give rise to terminally differentiated p63⁻ luminal cells [28]. It is believed that somewhere between these two steps probably arise the putative cancer precursor ("stem") cells, since many human cancers overexpress p63 [27], while most PCas and PCa cell lines (DU-145, LNCaP, and PC-3) have been reported to be p63⁻ [21]. It is also possible that the putative cancer "stem" cells arise directly from the prostate stem cells.

Other *in vitro* studies support at least partially the idea of the commitment of p63 to the stem cell lineage differentiation. Tokar *et al.* have isolated two cell lineages resembling normal prostate intermediate and stem cell populations. They observed that the intermediate cell lineage have expressed low p63, while stem cell lineage have expressed high p63 and cytokeratin 14 [22].

It is believed that PIA lesion is a precursor of prostate intraepithelial neoplasia (PIN) and PCa [16]. Our results have demonstrated that a part of the basal cells in PIA, which we designated as type I, have low p63, GSTP1, and high AMACR expressions. The loss of GSTP1 function in prostate cells triggers the vulnerability of these cells to suffering genomic damages mediated by both continuous chronic oxidant stress and dietary heterocyclic amine carcinogens [16]. We hypothesized that the lack of GSTP1 expression in type I basal cells suggest for a decreased DNA anti-damage protection.

AMACR plays a critical role in the peroxisomal β -oxidation of fatty acid molecules [10]. The first step of

the pathway in β -oxidation of branched chain fatty acids is an oxidation step catalyzed by acyl-CoA oxidases, with the products being oxidized substrate and hydrogen peroxide [10]. High AMACR expression suggests that generated hydrogen peroxide throughout peroxisomal β -oxidation directly influence on type I basal cells, which most likely have undergone changes of the genome.

Most of the prostate adenocarcinoma and high grade prostate intraepithelial neoplasia (HGPIN) cells do not express p63 suggesting that p63 may protect prostate epithelial cells against neoplastic transformation, possibly through a tumor suppressor function [17]. Indeed, the low p63 expression by type I basal cells, supports the notion that these particular cells are the direct targets for neoplastic alteration, since the decrease of p63 expression leads to increased cell migration and up-regulation of genes involved in invasion and metastasis and predisposes to a loss of epithelial and acquisition of mesenchymal characteristics [1]. p63 is found to be the molecular switch for initiation of the epithelial stratification program [6], which further supports the notion of its necessity for preserving proper epithelial glandular structure in the prostate.

The existing correlation between the expression levels of p63 and AMACR as well as between p63 and GST only in type I basal cells and the apparent lack of significant correlation in type II basal cells further supports this notion. This observation could be explained by the ability of p63 to influence the expression of AMACR and GST only in p63-low expression basal cells, where p63 protective function is diminished and relatively fewer factors come into place.

Although the origin of prostate carcinoma is still unknown [18], our investigation suggests that certain p63⁺ cells in the basal cell compartment are less protected and similar to the transit-amplifying actively proliferating cell lineage (p63⁺/PSCA⁻/AR⁻). Further complete loss of their p63 resemble the intermediate cell population as well as cancer cells, where null or low p63 corresponds to high levels of AMACR.

We propose that these type I basal cells are probably the first ones that undergo changes and are probably related to the subsequent tumorigenic alterations, beginning to lose their GSTP1 and p63, which is related with decreased DNA anti-damage protection and higher possibility of basal cell neoplastic transformation. Higher levels of AMACR imply a direct hydrogen peroxide influence on these cells.

The similarity of the antigen profile and localization of the type I basal cells makes it plausible that these cells are possibly the putative human prostate carcinoma stem cells from which PIN and PCa arise.

Further investigations are necessary to isolate this type of basal cells to confirm their role in the prostate

pathogenesis, which are in progress in our laboratory. Most importantly, stem cell research will provide novel therapies in the treatment of prostate cancer.

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